PRODUCTION BY ACTINOMYCETES OF (*S,S*)-*N,N'*-ETHYLENEDIAMINE-DISUCCINIC ACID, AN INHIBITOR OF PHOSPHOLIPASE C

Sir:

We have searched for specific inhibitors against various kinds of enzymes in culture filtrate of microbes and succeeded in isolating a variety of new compounds which inhibit enzymes specifically[1~3].

In the present study, we searched for inhibitors against phospholipases and discovered (*S,S*)-*N,N'*-ethylenediaminedisuccinic acid as a specific inhibitor against phospholipase C (PL-C, EC 3.1.4.3.) from the culture filtrate of an actinomycetes (MG417-CF17). Although the inhibitor we obtained was a known substance[4], because of the importance of its inhibitory actions we report the isolation and the characterization of this agent.

PL-C (*Clostridium perfringens*, 3.4 U/mg) was purchased from Calbiochem-Behring, La Jolla, California. The activity was measured as reported previously[6], and the concentration of the inhibitor required for 50% inhibition (IC_{50}) was determined.

The inhibitor was produced by shaken culture of MG417-CF17 in a medium containing 1.5% glycerol, 1.5% cotton seed meal, 0.3% NaCl, and 0.2% L-asparagine, pH 7.0 with 5 N NaOH before sterilization. The maximum production was attained on 2 days of culture at 27°C. The culture filtrate (10 liters) was boiled for 10 minutes at pH 8 and passed through a column of Amberlite IRA-400 (OH^-, 2 liters) pretreated with 0.01% aqueous ammonia. After washing with 0.01% ammonia, the inhibitor was eluted with 1 M ammonium chloride solution. The active eluate was applied to a column of Dowex 50WX8 (H^+, 0.8 liter), and the inhibitor was eluted with 2.5% ammonia after washing with water. The active eluate was concentrated under reduced pressure and the concentrate passed through a column of Amberlite CG-50 (H^+ type I). The effluent was concentrated under reduced pressure to a small volume and was kept in a refrigerator at pH 2.5 to give crude crystals of the inhibitor. The inhibitor was purified by repeated crystallization from acidic water to yield 1.7 g (colorless needles, mp 147~149°C).

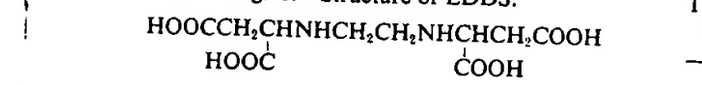
The inhibitor is soluble in alkaline water, slightly soluble in water but insoluble in methanol, ethanol, acetone, ethyl acetate and benzene. It gives positive color reactions with ninhydrin and DRAGENDORFF reagent, but negative with RYDON-SMITH reagent. The Rf value was 0.23 on Avicel TLC developed by 1-BuOH - AcOH - H_2O (2: 1: 1) and the Rm value (Ala 1.0) on high-voltage paper electrophoresis (3,500 V, 10 minutes, HCOOH - AcOH - H_2O, 1: 3: 36, pH 1.8) was 0.42. It showed only end absorption in the UV spectrum; $[\alpha]_D^{22}$ +31.2° (*c* 1, 6 N HCl); ^1H NMR (90 MHz, 20% ND_4OD, external TMS; δ=0) 2.94 (m, $-\overset{|}{C}H-CH_2-$), 3.24 (s, $-CH_2-$), 3.92 (q, $-\overset{|}{C}H-CH_2$); ^{13}C NMR (25 MHz, 20% ND_4OD, external TMS; δ=0) 42.0 (t, $-CH_2-\overset{O}{\overset{\|}{C}}-OH$), 47.2 (t, $-\overset{|}{C}H_2-N-$), 62.4 (d, $-CH-$), 180.4 (s, $-CH_2-COOH$) and 181.2 (s, $-\overset{|}{C}H-COOH$).

For the purpose of determination of molecular weight, the inhibitor was converted to its methyl ester (reflux in 5% HCl - MeOH at 60°C for 21 hours) and its molecular weight was determined. The high resolution mass spectrometric analysis of the methyl ester gave a molecular ion M^+ at *m/z* 348.1492 indicating the molecular formula $C_{14}H_{24}N_2O_8$: 348.1531. Thus the molecular weight of the inhibitor was shown to be 292 ($C_{10}H_{16}N_2O_8$), because the number of carbon atoms must be 10 and tetra methyl ester should be produced by the methylation mentioned. The elemental analysis was as follows; Calcd for $C_{10}H_{16}N_2O_8$: C 41.10, H 5.52, N 9.59, O 43.80. Found: C 41.45, H 5.63, N 9.12, O 43.13.

The NMR spectral analysis suggested the presence of two methylenes, one methine, and two carboxyl carbons. These signal represent half of a molecule.

After keeping the inhibitor in dimethyl sulfoxide for several hours, two methylenes (3.06 and 3.38 ppm), one ethylene (3.81 and 3.97 ppm) and two methines (4.47 and 5.50 ppm) were newly observed by ^1H NMR, and one carbonyl carbon (170.3 ppm) by ^{13}C NMR. Methylation of this compound by HCl - MeOH method gave a tri methyl ester, a structure supported by the observation of three methoxyl signals by ^1H NMR and mass spectrometry (M^+ at *m/z*: 316.1264, calcd for $C_{13}H_{20}N_2O_7$: 316.1268). These findings suggested that an asymmetrical change of the structure caused by dehydration between carboxyl and amino residues was occurring.

Fig. 1. Structure of EDDS.



From these results, the structure of the inhibitor was suggested to be *N,N'*-ethylenediaminedisuccinic acid (EDDS). NEAL and ROSE[4] reported the chemical synthesis of EDDS from L-aspartic acid and 1,2-dibromoethane: the molecular rotation, $[M]_D$, gave -680 ($[\alpha]_D -232.9°$) in aqueous 6 N hydrochloric acid. The natural product EDDS obtained from actinomycetes (MG417-CF17) showed however an opposite molecular rotation ($[\alpha]_D^{22}$ $+31.2°$ (*c* 1, 6 N HCl)).

In order to confirm the proposed structure and determine the configuration of the inhibitor, the X-ray crystallographic study of the copper complex was undertaken. The copper complex was produced by mixing the inhibitor and cupric carbonate in the water and crystallized from water (mp 210°C (dec)).

The X-ray data confirmed the structure of EDDS, and indicated *S* configuration at an asymmetric carbon (unpublished). Thus, the structure of the inhibitor was determined to be (*S,S*)-*N,N'*-ethylenediaminedisuccinic acid (Fig. 1) which was identical with the configuration reported by NEAL & ROSE[4] and SCARBROUGH & VOET[6].

We also prepared EDDS by the method of NEAL & ROSE[4] from L-aspartic acid and, 1,2-dibromoethane. The synthesized EDDS showed mp at 147~148°C, $[\alpha]_D^{22}$ $+28.3°$ (*c* 1, 6 N HCl), *Anal* Found: C 41.45, H 5.61, N 9.58, O 43.47, and was identical with the natural EDDS from actinomycetes (MG417-CF17) by IR and NMR spectrometries. It is concluded that both natural and synthesized EDDS had the same configuration since they had similar dextrorotatory optical activity. Thus we could not find evidence for the molecular rotation of EDDS found by NEAL & ROSE[4].

The inhibitory activity of EDDS against phospholipases are shown in Table 1 in comparison with EDTA. EDDS showed inhibition against phospholipase C and D, but not phospholipase A_2. The inhibition of EDDS is competitive with substrate. The *Ki* and *Km* values for phospholipase C are 8.56×10^{-6} M and 1×10^{-3} M, respectively. Intraperitoneal administration of 0.01~1 mg per mouse of EDDS suppressed delayed type hypersensitivity (DTH) and antibody formation. *In vitro*, it showed suppression of T and B blastogenesis. EDDS at 100 μg per ml had no antimicrobial activity. It has low toxicity: no deaths after intravenous injection of 500 mg per kg to mice.

Table 1. Inhibitory effects against phospholipase A_2, C and D.

Inhibitors*	IC_{50} (μg/ml)		
	PL-A_2	PL-C	PL-D
EDDS	>400	3.8	19
EDTA**	>400	0.43	204

* Inhibitors were dissolved in 0.05 M Tris-HCl buffer, pH 7.0.

** EDTA was used as tetra-sodium salt.

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